Stress assessment in captive greylag geese (Anser anser)

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Stress assessment in captive greylag geese (*Anser anser*)[1]

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ABSTRACT: Chronic stress—or, more appropriately, "allostatic overload"—may be physiologically harmful and can cause death in the most severe cases. Animals in captivity are thought to be particularly vulnerable to allostatic overload due to artificial housing and group makeup. Here we attempted to determine if captive greylag geese (*Anser anser*), housed lifelong in captivity, showed elevated levels of immunoreactive corticosterone metabolites (CORT) and ectoparasites in dropping samples as well as some hematological parameters (hematocrit, packed cell volume, total white blood cell count [TWBC], and heterophil:lymphocyte ratio [H:L]). All of these have been measured as indicators of chronic stress. Furthermore, we correlated the various stress parameters within individuals. Captive geese showed elevated values of CORT and ectoparasites relative to a wild population sampled in the vicinity of the area where the captive flock is held. The elevated levels, however, were by no means at a pathological level and fall well into the range of other published values in wild greylag geese. We found no correlations between any of the variables measured from droppings with any of the ones collected from blood. Among the blood parameters, only the H:L negatively correlated with TWBC. We examine the problem of inferring allostatic overload when measuring only 1 stress parameter, as there is no consistency between various measurements taken. We discuss the different aspects of each of the parameters measured and the extensive individual variation in response to stress as well as the timing at which different systems respond to a stressor and what is actually measured at the time of data collection. We conclude that measuring only 1 stress parameter often is insufficient to evaluate the well-being of both wild and captively housed animals and that collecting behavioral data on stress might be a suitable addition.

Key words: allostatic overload, captivity, corticosterone metabolites, greylag goose (*Anser anser*), hematological parameters, parasite

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INTRODUCTION

Stress is a real or perceived challenge to psychological or physiological stability (homeostasis; McEwen and Wingfield, 2003), and a stressor, for example, the transfer of wild animals into captivity, is anything that challenges it (Morgan and Tromborg, 2007). One response to an *acute* stressor is the release of glucocorticoids (**GC**). This temporary modulation is adaptive as GC have short-term protective effects. However, *chronic* elevation of GC may be physiologically harmful (McEwen and Wingfield, 2003). Therefore, GC have been used to determine health condition of individuals and populations (Gladbach et al., 2011).

Glucocorticoids influence other physiological parameters indicative of stress, such as blood parameters and parasite load. Decreased immune competence due to shifts in leukocytes may lead to increased parasite infestation or susceptibility to infection (e.g., Breuner, 2011). Therefore, quantifying parasites has been used to measure stress (Bortolotti et al., 2009). Notably, high parasitic burden modifies baseline GC levels (Mougeot et al., 2010), confounding cause and effect. Other parameters for stress assessment are the heterophil:lymphocyte ratio (**H:L**), which predicts the extent of the humoral immune response, or the hematocrit (**HCT**) in blood. Environmental stressors usually elevate heterophils and depress lymphocytes (Davis et al., 2008). Due to large variation caused by diverse natural factors (Fair et al., 2007), the relationship between HCT and condition in wild birds is not as easily estimated (Tripet and Richner, 1999).

Greylag geese (*Anser anser*) in this study always have been confined under adequate housing conditions; their group composition resembles wild goose flocks. Therefore, we hypothesized that, here, stress arising from confinement is negligible. Consequently, we asked if captive geese show stress levels similar to free-ranging greylag geese by evaluating their baseline immunoreactive corticosterone metabolites (**CORT**), comparing endoparasites of captive and wild geese, and examining the relationship between fecal and blood stress determinants.

MATERIALS AND METHODS

This project was approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC license 6383/6383A to J. Komdeur).

Study Areas and Study Populations

The study was conducted in the goose pen (68 m long by 60 m wide) of the animal care facility at the University of Groningen, Centre of Life Sciences (Groningen, The Netherlands). The fenced-in outdoor area is separated into 2 equal sections by wire mesh. Each of the 2 sections holds 1 pool (25 m long by 15 m wide) with permanent running water for swimming and drinking. The pens are covered with natural vegetation and contain several trees for shade. Geese are fed daily with commercial waterfowl food (Kasper Anseres 3 maintenance pellets and Kasper mixed grain with broken corn, with a pellet:grain ratio of 2:1; Arie Blok Animal Nutrition, Woerden, The Netherlands) year-round.

At the time of data collection (May 25–June 25, 2012), the captive flock consisted of 19 greylag and 49 barnacle geese (*Branta leucopsis*) separately housed in either section described above but in visual and vocal contact through the middle separation. All geese are individually marked with 3 colored leg bands and an aluminum band with a unique number. To prevent geese from flying away, the primary feathers of their right wing are clipped regularly during molt. Greylag geese at time of data collection consisted of 1 family (1 adult male and 1 adult female), 3 goslings (male, male, and female, hatched May 6, 2012), 3 heterosocial male–female pairs, 3 homosocial male–male pairs, and 2 single males (see Table 1 for details).

Dropping Samples – Collection

To compare corticosterone metabolites and parasite burden of the captive greylag goose flock with conditions in the wild, 2 of us (M. Sterenborg and I.B.R. Scheiber) collected fresh droppings from a wild greylag goose population approximately 25 km north of the University of Groningen campus at the National Park Lauwersmeer (**NPL**), northern Netherlands (35°21′74″ N, 6°14′75″ E), on July 6, 2012. National Park Lauwersmeer consists of grassy meadows and shallow open water in the former waterways and culverts of the Lauwers Sea. The Dutch Forestry Commission manages the greater part of the park. Scottish Highland cattle (*Bos taurus*), Konik horses (*Equus ferus f. caballus*), and sheep (*Ovis aries*) roam the pastures for grazing, which ensures that the large grassy meadows are not overgrown. In June 2012, approximately 1,500 greylag geese were present in the NPL (Kleefstra and De Boer, 2012). Geese in the NPL are not individually marked and shy away from humans. For dropping collection, therefore, we picked a secluded pond, at which we found approximately 80 geese, mainly consisting of families with their nearly fledged offspring. We carefully approached the geese so that they slowly retreated to the water body. We then walked the area where geese had been resting on either side of the pond and collected all fresh droppings. To ensure that we did not resample

Table 1. Nineteen captive greylag geese at the time of data collection, the hatch year, sex, social status, and various stress parameters measured. Empty cells indicate missing data

Goose ID[1]	Hatch year	Sex[2]	Social status[3]	Mean CORT[4]	Amidostomum anseris[5]	Coccidia[5]	HCT[6]	TWBC[7]	H:L[8]
8043840	2003	m	hetero	36.90	3	1	0.48	8,250	2.33
8049122	Before 2008	f	hetero	31.99	0	0	0.46	12,750	3.84
8049123	Before 2008	m	family	49.81	0	1	0.48		1.45
8049128	2009	m	homo	32.50	1	0	0.43	9,000	3.55
8049131	2009	f	family	22.53	3	0			1.09
8049134	2009	m	homo	55.35	3	1	0.47	5,250	2.95
8049135	2009	m	homo	58.60	1	1	0.48	8,250	3.16
8049136	2009	m	hetero	25.73	1	1	0.5	12,000	1.96
8049138	2010	f	hetero	103.32	2	2	0.48	6,000	2.21
8049139	2010	m	homo	53.15	2	3	0.44	13,500	1.30
8049142	2009	m	homo	44.29	0	0	0.51	9,750	2.78
8049143	2009	m	homo	44.15	0	1	0.5	6,750	2.35
8049146	2009	f	hetero	74.53	1	1	0.43	15,000	1.30
8049147	2009	m	hetero	43.02	0	0	0.44	16,500	1.67
8049149	2009	m	single	36.04	0	1	0.52	14,250	2.00
8049151	2010	m	single	63.48	1	0	0.5	9,750	2.90
8049153	2012	m	family		0	1	0.41	9,750	1.20
8049155	2012	m	family		0	0	0.37	15,000	0.98
8049156	2012	f	family		1	1	0.42	7,500	3.25

[1]ID = Individual Aluminium Band Number.

[2]m = male; f = female.

[3]family = male, female, and juvenile offspring; hetero = male–female pair; homo = male–male pair; single = unpaired.

[4]CORT = immunoreactive corticosterone metabolites; in nanograms CORT per gram feces.

[5]Parasitic *Amidostomum* and Coccidia infestation: 0 = negative, 1 = low, 2 = intermediate, and 3 = high.

[6]HCT = hematocrit; volume percent red blood cell in whole blood.

[7]TWBC = total white blood cell count.

[8]H:L = heterophil:lymphocyte ratio.

the same dropping repeatedly, we crushed the remnants after collection. In total, we collected 59 droppings from the wild geese (31 samples for corticosterone metabolite determination and 28 samples for parasite counts).

Dropping Samples – Analyses

For extraction of CORT, we collected a total of 26 dropping samples from 15 adult captive geese between May 25 and 31, 2012. We did not collect samples from the 3 juvenile geese, as they were not banded at that time and samples could not be individually assigned, as well as from 1 adult goose that only defecated into the pond. We attempted to collect a minimum of 2 samples per individual within 3 h after feeding, as CORT concentrations in individual samples are highly variable in geese (Scheiber et al., 2005a). This, however, was not always possible (range 1–3 and mean ± SD 1.67 ± 0.724). Individual samples from captive and wild geese were collected well after sunrise to avoid the effects of diurnal variation in corticosterone excretion (Schütz et al., 1997). As geese droppings consist of a mixture of uric acid and fecal matter, we collected and analyzed fecal matter with a minor uric acid fraction (Scheiber et al., 2009). The samples were frozen at –20°C within 1 h after collection and subsequently shipped on dry ice to the Department of Behavioral Biology at the University of Vienna (Vienna, Austria) for CORT determination. Dropping samples were analyzed using EIA with a group-specific antibody recognizing 5β,3α,11β-diol glucocorticoid metabolites developed for greylag geese (Möstl et al., 2005). Details of the procedure and cross-reactivities of this assay are published elsewhere (Frigerio et al., 2004). Concentration limits ranged from 15.15 to 103.32 ng CORT/g droppings in the captive geese and 4.33 to 49.03 ng CORT/g droppings in the wild geese. These values are well in range with previously published quantities in greylag geese (Scheiber et al., 2005b, 2009). Intra- and interassay CV were determined from homogenized pool samples. Mean intra- and interassay CV were 19.5 and 10.71% (<15%) in the captive geese and 19.5 and 11.84% (<25%) in the wild geese, respectively.

For determination of intestinal parasites, we collected 2 fecal samples per captive individual in the afternoon from the second to the July 5, 2012. Samples were stored immediately in a refrigerator at +6°C to prevent further development of eggs in the sample.

Samples for the wild geese were collected on July 6, 2012, in the NPL.

Dropping samples were shipped to the Department of Pathobiology at the University of Veterinary Medicine, Vienna, Austria, and examined by fecal flotation (sugar solution; specific gravity = 1.28). Assessment of intestinal parasites was done semiquantitatively in 4 categories: negative (no infestation; 0), low (1), intermediate (2), and high (3). Scores of 1 to 19 Coccidia, for example, approximately equate to low infestation, 20 to 30 to intermediate infestation, and >30 to high infestation, whereas the number of oocysts refers to all found under 1 microscope slide.

Blood Samples

To collect blood samples, the captive geese were driven into a funnel trap that is permanently set in the goose pens. Fourteen of the adult geese were sampled on June 1, 2012, and the 2 parental adults and 3 goslings were sampled on June 25, 2012. At this point, goslings are large enough that they can be individually marked. For blood sampling, 1 goose at a time was caught from the funnel trap and brought inside a building adjacent to the goose pens. Here the tibial vein was punctured with a sterile (24 gauge) needle and 2 heparinized microhematocrit capillaries (75 mm/75 µL) were filled. We prepared 2 blood smears from each sample and submitted those to the Central Laboratory of the University for Veterinary Medicine in Vienna for a differential blood count. The HCT capillaries were then sealed with plasticine at the bottom and centrifuged (Hermle Microliter Centrifuge Z-233 M-2 with hematocritrotor 220.58 V08; Hermle Labortechnik GmbH, Wehingen, Germany) at 6,000 rpm relative centrifugal force = 370.282) for 6 min to separate blood cells and plasma. Red blood cells and plasma were measured with calipers to the nearest 0.5 mm. Hematocrit or packed cell volume (**PCV**) was calculated according to the following formula: PCV (%) = (length of the red cell column/length of plasma + red cells) × 100.

Blood smears were stained with a Romanowsky type stain (Haemaquick; Eberhard Lehmann GmbH, Salzburg Austria) and microscopically evaluated. Total white blood cell count (**TWBC**) was estimated by counting the white blood cells in five 400x microscopic fields. For the differential blood count, 100 white blood cells were differentiated into heterophilic, eosinophilic, or basophilic granulocytes, monocytes, and lymphocytes under 1,000x oil immersion. Results were given in percentages. Blood smears from 2 geese were of bad quality and TWBC could not reliably be determined. As TWBC did not differ significantly between the 2 slides we had produced from each individual (Wilcoxon signed rank test: $Z = 0.106$, $P = 0.946$, $n = 17$), we calculated individual means from the 2 slides for all cell types. Heterophils and lymphocytes were the most abundant cells in the samples and account for 57.4 ± 9.3 and 30.5 ± 8.8% (mean ± SD), respectively. To assess the relative quantity of the various leukocytes, we calculated the H:L from the means per cell type per individual.

Data Analyses

Data were analyzed using SigmaPlot 11.0 (Systat Software, San Jose, CA). All tests are given 2-tailed with a level of significance of $\alpha \leq 0.05$. We tested for normality of the data distribution with Shapiro–Wilk tests and applied appropriate parametric or nonparametric tests whenever applicable. If variances of normally distributed data were not equal, we tested with the appropriate nonparametric tests. For a comparison of fecal parameters between captive and wild geese, we performed Mann–Whitney rank sum tests (**M-W**). Whereas CORT is a continuous variable, we considered parasitic infestation (*Amidostomum anseris* or Coccidia species, respectively) a binomial variables (yes/no), as only a few geese fell into intermediate and high categories. We performed Pearson product moment correlations (Pearson's *r*) for correlations between blood parameters (HCT, TWBC, and H:L), and in cases of significant correlations we performed a linear regression analysis. For relating fecal to blood parameters, we performed Pearson's *r* for CORT, HCT, TWBC, and H:L. For parasitic infestation, we performed 1-way ANOVA when comparing parasites with blood parameters. To have large enough samples sizes also in the intermediate and high infestation levels, we collapsed our original 4 infestation levels to 3 (no infestation [0], low [1], and intermediate and high [2]).

RESULTS

Comparing Fecal Parameters between Captive and Wild (National Park Lauwersmeer) Greylag Geese

Immunoreactive corticosterone levels in adult captive male and female greylag geese did not differ (M-W: $U = 23.000$, $P = 0.952$, $n_{males} = 17$, $n_{females} = 4$). Therefore, we pooled data for further analyses. However, captive geese showed higher CORT levels than NPL geese (M-W: $U = 97.000$, $P = 0.001$, $n_{captive} = 16$, $n_{NPL} = 29$; Fig. 1). Furthermore, there was no sex difference in captive male and female greylag geese for endoparasitic infestation (M-W: $U = 34.500$, $P = 1.0$, $n_{males} = 14$, $n_{females} = 5$), but more captive geese tested positive (15/19) relative to NPL geese (8/28; M-W: $U = 132.000$, $P < 0.001$, $n_{captive} = 19$, $n_{NPL} = 28$).

Coccidia (*Eimeria* spp.) and the nematode *Amidostomum anseris* (gizzard worm) were the most

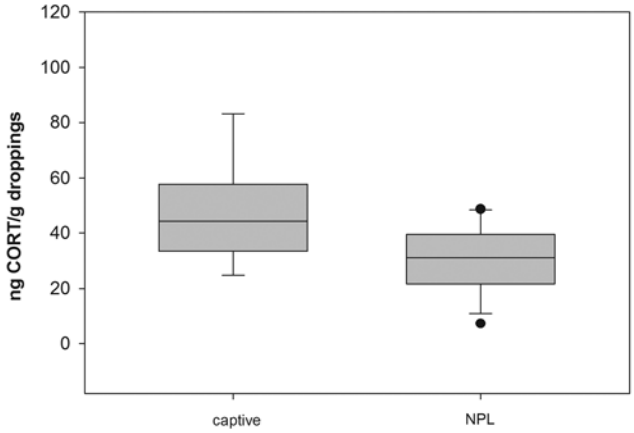

Figure 1. Immunoreactive corticosterone metabolite levels (in ng per g droppings) of captive and wild (National Park Lauwersmeer [NPL]) greylag geese. Median values of wild geese are significantly lower than those of the captive geese. Box plots show medians and quartiles, whiskers show 10th and 90th percentiles, and circles show 5th and 95th percentiles.

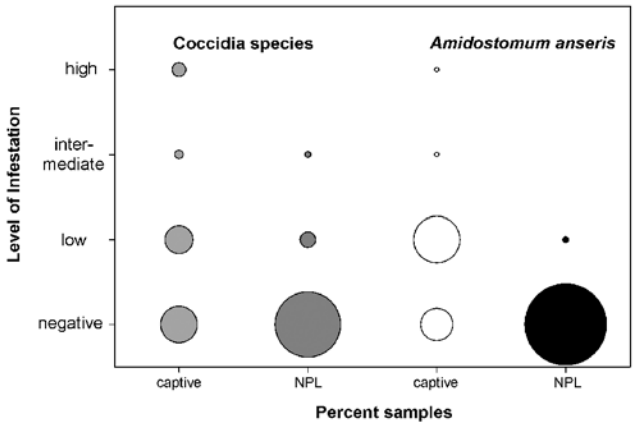

Figure 2. Percent dropping samples, which fall into a given level of infestation with either Coccidia species (left panel, light and dark gray circles) or *Amidostomum anseris* (right panel, white and black circles) of captive (n = 19) or wild greylag geese from the National Park Lauwersmeer (NPL; n = 28). The size of the circle specifies the number of individuals that fall in any given infestation level.

common intestinal parasites in the samples. These 2 parasites were also the most common in a study on free-living, human-habituated (Wascher et al., 2012) and feral greylag geese (Woog et al., 2011). Sporadically, we found samples of various species of other nematodes (round worms) in the samples. In the droppings of 2 captive geese, we found eggs of *Syngamus trachea* (gape worm), 1 captive goose sample contained eggs from *Trichostrongylus* spp. (strongyle worm), and in the droppings of 1 wild goose we found eggs of *Capillaria* spp. However, as sample sizes here were too small, we did not consider those parasites for later analyses. We found that captive geese were infested to a higher degree with the 2 most common intestinal parasites, Coccidia species (M-W: U = 169.000, P = 0.016, $n_{captive}$ = 19, n_{NPL} = 28) and *Amidostomum anseris* (M-W: U = 115.000, P < 0.001, $n_{captive}$ = 19, n_{NPL} = 28; Fig. 2) than the NPL geese. Although the 2 groups were significantly different, overall infestation was low also in captive greylag geese, where most individuals were not at all infested or at low levels of infestation. Immunoreactive corticosterone metabolite values did not differ between infested and non-infested geese. This was the case in captive geese (M-W: U Amidostomum = 17.000, P = 0.175, $n_{captive\ geese\ infested}$ = 10, $n_{captive\ geese\ not\ infested}$ = 6; U Coccidia species = 21.000, P = 0.357, $n_{captive\ geese\ infested}$ = 10, $n_{captive\ geese\ not\ infested}$ = 6) as well as NPL geese alike (U Amidostomum = 25.000, P = 0.964, $n_{NPL\ geese\ infested}$ = 2, $n_{NPL\ geese\ not\ infested}$ = 26; U Coccidia species = 70.000, P = 0.874, $n_{NPL\ geese\ infested}$ = 7, $n_{NPL\ geese\ not\ infested}$ = 21).

Comparing Hematological Parameters (Hematocrit, Total White Blood Cell Count, and Heterophil:Lymphocyte Ratio) within Captive Geese

As males and females did not differ in the 3 blood parameters we investigated (M-W: U HCT = 17.000, P = 0.55; U TWBC = 23.500, P = 0.86; U H:L = 32.500, P = 0.85, n_{males} = 14, $n_{females}$ = 5 for all 3 tests, respectively), we pooled data from males and females for further analyses. Between the 3 measured hematological parameters determined from blood, we found that TWBC is inversely related to the H:L (simple linear regression: R^2 = 0.276, n = 17, SE = 0.765, df = 1, F = 5.708, P = 0.03; Fig. 3). Neither TWBC and HCT (Pearson's r: r = –0.300, P = 0.241, n = 17) nor H:L and HCT (Pearson's r: r = 0.312, P = 0.207, n = 18) were significantly correlated.

Comparing Fecal and Hematological Parameters within Captive Geese

We found no relationships between any of the fecal parameters (CORT, *Amidostomum anseris* load, and Coccidia species load) and any of the blood parameters (HCT, TWBC, and H:L; Table 2).

DISCUSSION

Being capable of responding to stressful situations, both in the short and long term, is essential for health as chronic stress—or more appropriately termed allostatic overload—is harmful and may even result in death (McEwen, 2000; Sapolsky et al., 2000; Bartolomucci et al., 2005; Bartolomucci, 2007; Cyr et al., 2007; Romero and Butler, 2007). Animals held in captivity are often presumed to be susceptible to allostatic overload due

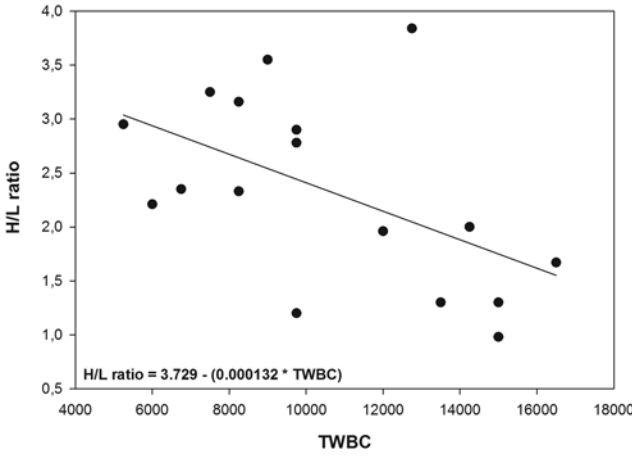

Figure 3. The heterophil to lymphocyte ratio (H:L) is negatively correlated with number of white blood cells (simple linear regression: $R^2 = 0.276$, $n = 17$, SE = 0.765, df = 1, $F = 5.708$, $P = 0.03$). The regression equation is given. TWBC = total white blood cell count.

to, for example, housing conditions or unnatural group composition (Terio et al., 2004). In response to allostatic overload, a variety of physiological and/or immunological response systems may be launched depending on the nature of the stressor. Each system, however, has its own inherent costs, as initiating any one of them is usually associated with reciprocal tradeoffs (Sterling, 2004). Therefore, each response system or the combination of various systems that is turned on in response to a stressor should be balanced against the expected range of demand to achieve the most effective but least costly way to return to homeostasis. Here we investigated various parameters indicative of stress both in wild and captive greylag geese to determine 1) if captive animals are "chronically stressed" and 2) if various physiological parameters involved in response to stress are correlated.

Are Captive Greylag Geese Chronically Stressed?

A comparison of corticosterone metabolites as well as parasite infestation collected from droppings of wild and captively housed greylag geese revealed that, indeed, captive geese showed significantly higher CORT levels than did wild geese from the NPL. The only CORT data set, which is to some extent comparable to this work, as the applied methodology was similar, was done on free-ranging greylag goose families in Grünau, Austria. Immunoreactive corticosterone metabolite level ranges of the captive geese in our study fall well into the ranges presented in this work (captive geese, range 15.15–103.32, and Grünau geese, range 0.15–250.50); however, mean values in the captive geese resemble mean values collected under an experimentally stress-induced condition in Grünau (captive geese: 48.46 ± 20.26 ng CORT/g droppings; Grünau geese: $\text{mean}_{\text{control}}$ 25.13 ± 3.06 ng CORT/g droppings and $\text{mean}_{\text{stress}}$ 43.05 ± 9.20 ng CORT/g droppings; see Scheiber et al., 2005a, for details). It should be mentioned that data in Grünau were collected only from families, which have the lowest levels of CORT relative to any other social categories, that is, pairs and singles in the flock (S. Kralj-Fišer, Konrad Lorenz Forschungsstelle, Grünau, Austria, and I.B.R. Scheiber, personal communication), due to benefits of social support (for a review, see Scheiber et al., 2013, Chapter 9). Therefore, although corticosterone metabolites are elevated in captive greylag geese, they are still reasonably low and do not indicate chronically high stress levels. This also implies that the difference between the NPL geese and the captive individuals is due to the fact that NPL geese have markedly low levels (range 4.33– 49.03 and mean 30.12 ± 11.70 ng CORT/g droppings) rather than CORT levels of the captive geese being extremely high.

Analyses of fecal droppings also revealed that, relative to wild geese, a higher percentage of captive geese were infested with the gizzard worm (*Amidostomum anseris*) and Coccidia species. Fifteen of the 19 cap-

Table 2. Results of correlations between fecal and hematological stress parameters (in bold). We performed Pearson product moment correlations (Pearson's r) for continuous variables (immunoreactive corticosterone metabolites [CORT] versus hematocrit [HCT], total white blood cell counts [TWBC], and heterophil:lymphocyte ratio [H:L]) and 1-way ANOVA for comparisons of continuous and categorical variables (*Amidostomum* spp. and Coccidia). In all comparisons, test statistic (r or F), significance values (P), and samples sizes are given.

	Haematological Parameters		
Fecal parameters	HCT	TWBC	H:L
CORT (Pearson's r)			
r	–0.116	–0.284	–0.0640
P	0.680	0.325	0.814
n	15	14	16
Amidostomum anseris			
F	0.041	1.768	1.218
P	0.960	0.207	0.322
df	2	2	2
n_{negative} [1]	8	8	8
n_{low} [1]	6	6	6
$n_{\text{intermediate–high}}$ [1]	5	5	5
Coccida species			
F	0.326	0.963	0.394
P	0.727	0.406	0.681
df	2	2	2
n_{negative}	7	7	7
n_{low}	10	10	10
$n_{\text{intermediate–high}}$	2	2	2

[1] n_{negative} = ; no infestation n_{low} = 1-19; $n_{\text{intermediate–high}}$ = 20-30; high ≥ 30.

tive geese tested positive to infestation by parasites (both parasites, 8/19; Coccidia species only, 4/19; and *Amidostomum anseris* only, 3/19). Sixty-three percent of geese, which were infected with gizzards worms, and 58% of geese, which were infected with Coccidia, fit well into the range of infected greylag geese of the free-ranging Grünau flock, where 87.1% of geese were infected with *Amidostomum anseris* and 50.3% with coccidian oocysts (Wascher et al., 2012). Our findings revealed that merely a minority of captive geese was either moderately or heavily infested with *Amidostomum anseris* (33%, 5/15 geese) or Coccidia species (13%, 2/15 geese), and the significant difference between NPL and captive geese is, again, more likely due to very low levels in the Lauwersmeer geese rather than captive geese showing high infestation. Alternatively, captive geese may show higher parasite load relative to wild geese because there is a higher risk of reinfection due to confined housing conditions, where avoiding potentially contaminated areas is not possible. The later idea, however, is confuted by the fact that the free-ranging Grünau geese are also infested with parasites to a similar extent.

There is some evidence that parasites activate their hosts' immune system (e.g., Esparza et al., 2004; Arriero et al., 2008; King et al., 2011; van de Crommenacker et al., 2012) and that this, in turn, affects the hypothalamic–pituitary–adrenal axis (Raouf et al., 2006), but not always (Carlberg and Lang, 2004; Monello et al., 2010). Our study also provides no evidence that parasite infestation modulates corticosterone metabolite excretion.

It has been shown that in birds, various factors may influence the modulation of both corticosterone and parasites, for example, seasonality (Dawson and Howe, 1983; Wingfield et al., 1994; Romero et al., 1998a,b; Romero and Wingfield, 1998; Naphade, 2013; Grema et al. 2014), inclement weather (Frigerio et al., 2004), or developmental stage (Swoboda, 2006). Additionally, most passerines downregulate baseline and stress-induced corticosterone release during molt to prevent that the protein catabolic activity of corticosterone does not interfere with protein deposition essential for feather growth (Romero, 2002; Romero et al., 2005). Endoparasites in greylag geese also seem lowest during our sampling period, and this, at least in part, may be predominantly explained by climate (Woog et al., 2011; Wascher et al., 2012). These findings support our idea that for our captive flock stress experienced from confinement is insignificant, as we collected data at a time when wild geese generally show extremely low levels in corticosterone profiles and parasite burden at that point.

Are Various Stress Parameters Correlated?

Hematological Measures (Total White Blood Cell Count, Hematocrit, and Heterophil:Lymphocyte Ratio). Hematology has long provided some valuable indicators of individual health and condition (Masello et al., 2009). Total white blood cell count values of captive geese (range 5,250–16,500, mean ± SE 10,544 ± 0.84) are consistent with data collected from wild, healthy juvenile and adult Nigerian ducks (*Anas platyrhynchos*; Olayemi et al., 2003) and Canada geese (*Branta canadensis*; Campbell, 2012, Table 19.4), the only 2 other waterfowl species for which we could find comparable data. Total white blood cell count values in birds are found to be between 5,000 and 15,000 but may exceed 25,000, depending on a variety of factors, including, for example, age or size of a species.

Furthermore, the relative proportions of different types of circulating leukocytes have proven useful as indicators of stress. The stress-related increase of H:L has been shown by many studies and seems now a well-established method to determine a composite measure of the stress response (for a recent review, see Davis et al., 2008; e.g., Dhabhar, 2002; Gladbach et al., 2010; Gross and Siegel, 1983; Maxwell, 1993; Vleck et al., 2000). In the majority of birds, including various species of waterfowl (black-necked swans [*Cygnus melanocoryphus*; Artacho et al., 2007], mute swan [*Cygnus olor*; Dolka et al., 2014], upland geese (*Chloephaga picta leucoptera*; Gladbach et al., 2010], and 10 species of waterfowl in 4 genera [Matson et al., 2006]), lymphocytes are the most prominent leukocyte population in circulation. There are some noticeable exceptions, however, including geese, in which heterophils are the most abundant cell type (Maxwell and Robertson, 1998). This is what we also found here. By definition, this causes the H:L to range above 1 (range 0.98–3.84, mean ± SE 2.25 ± 0.204). The rule of thumb, where values above 0.8 are indicative of high degrees of stress in most birds (Maxwell and Robertson, 1998), therefore, does not apply to geese and conclusions with respect to the stress response are not easily interpreted as data from wild conspecifics are lacking. Furthermore, the catching and blood collection procedure alone can induce changes in the leukogram (Gilor and Gilor, 2011).

Fecal and Hematological Measures (Corticosterone Metabolites, Parasite Load, Total White Blood Cell Count, Hematocrit, and Heterophil: Lymphocyte Ratio). We found no relationships of various fecal and hematological parameters, which are indicative of stress in captive greylag geese. Our lack of finding correlations between these parameters may refute our prediction that individuals who are stressed extensively over a prolonged period of time should show some consistency

with respect to the determinants of stress we investigated. What this might mean is that in our setting, captivity alone is not a strong enough stressor and that to find possible correlations between the various parameters, we would have to challenge the geese rigorously and possible also over an extended period of time. On the other hand, it is possible that our physiological measures may in fact work autonomously and, therefore, are indeed not correlated. This is similar to findings by Matson et al. (2006), who also found no simple relationships between different determinants of the immune response, that is, plasma and leukocytes assays, both on an individual and species level. Finally, it needs to be kept in mind that certain measures may fluctuate seasonally, may differ between sexes or age classes, and often may vary widely between species, which makes it difficult to draw conclusions (see our example of the H:L criterion of stress levels mentioned above). As heterophils are an important cellular component of the innate immune response, they generally are the first ones to respond to remove pathogens and produce signals to attract other leukocytes. Therefore, both positive and negative correlations between plumage brightness and H:L were used as signaling good health in birds (for details and a review, see Davis et al., 2008). In some studies, an increase in number of heterophils was interpreted as an indicator of stress and illness, whereas others deduced this to be an improved ability to respond to infection. By the same token, another caveat is that a reduced number of lymphocytes may signal an active stress response or the absence of a parasitic infection (Davis et al., 2008). Finally, there are other issues to be considered as well. For instance, cell counts from blood smears give information about only the relative amount of circulating heterophils and leukocyte types at the time of data collection but not how many of them are stored in other body parts and would be available to an individual in case of need (Davis et al., 2008). In summary, the various parameters measure entirely different aspects of the stress response, and there is extensive individual variation in response to stress (Vleck et al., 2000; Dhabhar and McEwen, 2001) as well as the timing in which the various systems respond as well as what is being measured at the time of data collection. For example, it has been repeatedly shown that circulating corticosterone levels and the H:L were not correlated (e.g., Maxwell, 1993; Vleck et al., 2000; Müller et al., 2011), which is most likely due to the time lag, that is, 30 min to days, associated with the initial leukocyte response to stress relative to a much faster increase of corticosterone in blood, that is, >3 min (Davis, 2005; Davis et al., 2008; Cirule et al., 2012). Therefore, measuring corticosterone metabolites might be a more suitable method, as also here there is a time lag, depending on gut passage time, between a perceived stressor and the time, when the respective metabolites appear in droppings. Studies in which both corticosterone metabolites from droppings and H:L were determined simultaneously are, to our knowledge, quite rare (Schulz et al., 2005; Jankowski et al., 2010), and we are not aware of any work in which these 2 measures were later directly compared. For instance, Jankowski et al. (2010) investigated the effects of an insecticide on the immunological response to West Nile virus in laboratory-housed chickens (*Gallus gallus domesticus*), where some individuals were given exogenous corticosterone. They found that this exposure increased both the H:L and corticosterone metabolites but did not report whether these 2 were correlated.

In summary, various parameters have been used to determine if animals are stressed, but frequently only 1 type of measurement is taken. Most often, this is the amount of circulating stress hormones, but other hematological or fecal stress response determinants have been used as well. As Evans et al. (2013) point out, various parameters, however, do not necessarily follow the same response pattern. In their study on actual and perceived physiological stress reactivity, all but 1 correlation yielded nonsignificant results between stress response indices in human children and adolescents (Evans et al., 2013). Similarly, in our study, we also found only 1 significant negative correlation between 2 hematological variables that are modulated during the stress response, that is, TWBC and the H:L, and no correlations between data collected from blood and droppings.

In conclusion, there does not seem to be a consistent pattern overall in various endocrinological and hematological parameters collected from blood and droppings, which in one way or another are known to be modulated during chronic stress. Therefore, neither measure alone seems to reliably predict chronic stress in animals and makes inferences complicated. This is especially the case for captive animals, if data from their wild counterparts are lacking. Here, behavioral observations might be a trustworthy alternative to measure well-being. Ideally, when interested in studies investigating stress levels in animals, a combination of various factors may be advisable (Wielebnowski, 2003), as the informative value of one measure may just not be enough to make the correct assessments of an animal's health and welfare. With respect to the captive greylag goose flock we investigated, our findings suggest that although some parameters are suggestive of higher stress levels relative to 1 wild greylag goose population, they are by no means unusual and are well within the range of other waterfowl species, including 1 other flock of semitame greylag geese. Due to the habituation of the Groningen geese to their human caretakers, we consider them a suitable model for experimental manipulation

under controlled conditions, particularly in answering questions related to stress coping mechanisms.

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